Matrix metalloproteinase-1 and -9 activation by plasmin regulates a novel endothelial cell-mediated mechanism of collagen gel contraction and capillary tube regression in three-dimensional collagen matrices

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SUMMARY

Here, we describe a new function for plasmin and matrix metalloproteinases (MMPs), which is to regulate the regression of capillary tubes in three-dimensional extracellular matrix environments. Using a well-described capillary morphogenesis system in three-dimensional collagen matrices, a new model of capillary regression has been established by adding plasminogen to the culture medium. Plasminogen is converted to plasmin by endothelial cell plasminogen activators which then induces matrix metalloproteinase-dependent collagen gel contraction and capillary regression. Plasminogen addition results in activation of MMP-1 and MMP-9, which then results in collagen proteolysis followed by capillary regression. The endothelial cells undergo apoptosis following gel contraction as detected by flow cytometric analysis as well as by detectable caspase-3 cleavage and caspase-dependent cleavage of the actin cytoskeletal regulatory protein, gelsolin. In addition, directly correlating with the contraction response, tyrosine phosphorylation of p130cas, an adapter protein in the focal adhesion complex, is observed followed by disappearance of the protein. Proteinase inhibitors that block MMPs (TIMP-1 or TIMP-2), plasminogen activators (PAI-1) or plasmin (aprotinin) completely block the gel contraction and regression process. In addition, chemical inhibitors of MMPs that block capillary regression also block MMP-1 and MMP-9 activation suggesting that a key element in this regression response is the molecular control of MMP activation by endothelial cells. Blocking antibodies directed to MMP-1 or MMP-9 interfere with capillary regression while blocking antibodies directed to PAI-1 accelerate capillary regression suggesting that endogenous synthesis of PAI-1 negatively regulates this process. These data present a novel system to study a new mechanism that may regulate regression of capillary tubes, namely, plasmin and MMP-mediated degradation of extracellular matrix.

Key words: Matrix metalloproteinase, Plasminogen, Regression, Endothelial cell, Morphogenesis, Collagen gel contraction

INTRODUCTION

Considerable work is currently ongoing to investigate the molecular mechanisms that regulate new blood vessel formation (i.e. angiogenesis) and the separate process of blood vessel regression (Hanahan and Folkman, 1996; Hanahan, 1997; Folkman, 1997). Both in vitro and in vivo studies have identified many classes of molecules that are relevant to these events such as angiogenic cytokines, integrins, extracellular matrix, proteinases and proteinase inhibitors (Vernon and Sage, 1995; Senger, 1996; Pepper et al., 1996; Werb et al., 1999; Haas and Madri, 1999). Recent work has identified several molecules that can induce regression of angiogenic vessels within tumors such as fragments of plasminogen (Plg) (i.e. angiostatin) and collagen type XVIII (i.e. endostatin) (O'Reilly et al., 1994; O'Reilly et al., 1997; Sage, 1997) or antagonists of integrin function such as cyclic RGD peptides and anti-integrin monoclonal antibodies (Brooks et al., 1994). In addition, withdrawal of vascular endothelial growth factor from tumor angiogenic vessels induces vessel and tumor regression (Benjamin and Keshet, 1997; Benjamin et al., 1999). Angiopoietins, by controlling endothelial cell (EC)-vascular smooth muscle cell interactions, also appear to regulate a balance between vessel growth and regression (Hanahan, 1997; Holash et al., 1999). Angiopoietin-1 knockout mice have revealed abnormal vascular structures that may be causally related to altered EC-extracellular matrix (ECM) interactions, although no definite mechanism for these findings has yet been reported (Suri et al., 1996).

Many studies have shown that ECs induce the expression of various proteolytic enzymes during angiogenic events (Herron et al., 1986; Mignatti et al., 1989; Fisher et al., 1994; Iruela-Arispe et al., 1995; Mignatti and Rifkin, 1996; Moses et al., 1996; Pepper et al., 1996; Haas et al., 1998; Hiraoka et al.,

1998). Relevant proteinases in these events include Plg activators (PAs), and matrix metalloproteinases (MMPs) (Pepper et al., 1996; Carmeliet and Collen, 1998; Nagase and Woessner, 1999; Werb et al., 1999). Their role in this process has been thought to be related to ECM breakdown, a step apparently required for EC morphogenesis (Mignatti et al., 1989; Fisher et al., 1994; Hiraoka et al., 1998). In support of their role in EC morphogenesis is that proteinase inhibitors, such as TIMPs, have been shown to inhibit angiogenesis (Moses et al., 1990; Moses, 1997). Recent data strongly implicates the role of membrane type MMPs (MT-MMPs) in EC and other cellular invasive and morphogenic behaviors in three-dimensional extracellular matrices (Hiraoka et al., 1998; Hotary et al., 2000). In contrast, secreted MMPs, such as MMP-1, MMP-2 and MMP-9, were not shown to play a role in collagen gel invasion and morphogenesis of MDCK cells in this latter study (Hotary et al., 2000). Thus, despite the induction of secreted MMPs during many types of cellular morphogenesis, their role in these events is unclear.

A very interesting question, and one that has been only minimally investigated, is whether or not some EC-derived proteinases may be induced during angiogenesis to regulate the process of vascular regression rather than morphogenesis. During angiogenesis, the final pattern of blood vessel formation is governed by the concurrent and dual regulation of EC morphogenesis and regression (Ausprunk et al., 1978; Clark, 1996; Folkman, 1997). This formation and regression of blood vessels is characteristically observed within granulation tissue during wound healing responses or during the female reproductive cycle (Moses et al., 1996; Clark, 1996; Madri et al., 1996). The mechanisms regulating vascular regression during these events are unknown, however, past and recent literature has shown that other tissue regression phenomena, such as mammary gland involution following the cessation of lactation, involves ECM proteolysis (Talhouk et al., 1992; Sympson et al., 1994; Werb et al., 1996). These proteolytic events lead to mammary epithelial cell apoptosis and regression of mammary epithelial ducts (Boudreau et al., 1996) secondary to an imbalance of proteinase activity relative to proteinase inhibitor activity (Werb et al., 1996). Since the ECM comprises the scaffold on which tissues assemble, differentiate and survive, disruption of this scaffold can result in tissue regression through mechanisms such as apoptosis (Meredith et al., 1993; Boudreau et al., 1996). These concepts, generated from studies of mammary gland involution, are likely to be of general importance to other types of cellular regression responses including those involving the vasculature.

Here, we present an in vitro model of capillary tube regression that is directly related to these latter concepts and that involves ECM proteolysis. We report on a novel function for plasmin and matrix metalloproteinases in the context of EC function, which is to regulate vascular regression in three-dimensional extracellular matrix environments. The addition of plasminogen (Plg) to a model of capillary morphogenesis in three-dimensional collagen matrices results in marked EC-mediated collagen gel contraction and regression of capillary tube networks. This occurred through the conversion of Plg to plasmin through EC plasminogen activators (PAs), which then activated matrix metalloproteinases (MMPs). Proteinase inhibitors or blocking antibodies directed to MMPs interfered with this contraction/regression event. Evidence for EC apoptosis was observed during these events by caspase-dependent cleavage of the actin regulatory protein, gelsolin. Blocking antibodies to PAI-1 markedly accelerated the MMP-dependent regression response. These data support the concept that plasmin and MMPs, such as MMP-1 and MMP-9, can participate in endothelial cell-mediated collagen gel contraction and regression of capillary tubes.

MATERIALS AND METHODS

Human umbilical vein endothelial cells were obtained from Clonetics Corp. (San Diego, CA), and were grown according to the supplier or as described (Maciag et al., 1979). ECs were used in our experiments from passages 2-6. The EC morphogenesis assay in three-dimensional collagen gels was performed as described using either gels at 3.75 or 5 mg/ml of collagen type I (Davis and Camarillo, 1996; Salazar et al., 1999). Glu-Plg (American Diagnostica, Greenwich, CT) was added at various concentrations to the serum-free culture medium which contained Medium 199, a 1:200 dilution of the Reduced Serum supplement II, 50 μg/ml of ascorbic acid, 50 ng/ml of phorbol ester, and 40 ng/ml of recombinant VEGF-165 and 40 ng/ml of FGF-2. Recombinant TIMP-1 and TIMP-2 as well as monoclonal antibodies directed to MMP-1 (Blocking-COMY 4A2) (Non-blocking-III7) (Birkedal-Hansen et al., 1998), MMP-2 (CA-4001) (Margulies et al., 1992), MMP-9 (GE-213) (Soini et al., 1994) were from Chemicon Corp (Temecula, CA). Recombinant PAI-1, the MMP inhibitor GM6001, and carboxypeptidase B were from Calbiochem (La Jolla, CA) and aprotinin was from American Diagnostica. Another monoclonal antibody directed to MMP-1 (41-1E5) was obtained from Oncogene Research Products (Cambridge, MA) while an anti-MMP-3 (4B7.3), and anti-gelsolin monoclonal antibodies were obtained from Sigma Corp. (St Louis, MO). Monoclonal antibodies to caspase-3, p130cas and phosphotyrosine (PY20) were obtained from Transduction Laboratories (Lexington, KY). A rabbit polyclonal antiserum directed to the MMP-9/TIMP-1 complex purified from HL-60 cell promyelocytic leukemia cells was utilized as previously described (Davis and Martin, 1990). Sheep polyclonal antibodies directed to PAI-1 and PAI-2, which block function, were purified using protein G-sepharose and were obtained from Chemicon Corp. Rat tail collagen type I was prepared as described (Bornstein, 1958). Fluorescein isothiocyanate, phorbol ester, Protein G-Sepharose and α2 macroglobulin were obtained from Sigma Corp.

Plasminogen-induced endothelial cell contraction of collagen gels

EC cultures were prepared as described above and as previously described (Davis and Camarillo, 1996) with or without the addition of Plg. In most cases, Plg was added at 2 μg/ml at the initiation of culture. Cultures were fixed at 48 hours with 3% glutaraldehyde in PBS, pH 7.5, for at least 30 minutes prior to additional manipulation. In some cases, cultures were stained with 0.1% toluidine blue in borax and then destained prior to visualization and photography. Contracted gels were measured by determining the diameter of the gel in two representative perpendicular axes. The average diameter was then used to calculate the area of the gel which used a formula to calculate the area of an ellipse. In some experiments, protease inhibitors, or anti-MMP antibodies were added at the indicated concentrations in the figure legends. In some experiments, contracted or non-contracted gels were digested with bacterial collagenase and the resulting cell pellet was fixed with 70% ethanol. The cells were then further processed for flow cytometric apoptosis analysis by staining with propidium iodide as described (Maxwell and Davis, 2000a; Maxwell and Davis, 2000b). In other experiments, conditioned medium was collected to examine differential proteinase expression over a 72 hour period of culture. Conditioned medium samples were run on 8.5%

SDS-PAGE gels, and after protein transfer to PVDF membranes, western blots were performed as described (Salazar et al., 1999) using chemiluminescence to examine protein expression patterns. In addition, gelatin zymograms were performed in the presence or absence of Plg added in the SDS-PAGE gel as described (Heussen and Dowdle, 1980; Davis and Martin, 1990). To visualize proteinase bands, 10 mM Tris-HCl, pH 7.5, with 5 mM $CaCl_2$ was used for zymograms without added Plg while gels containing Plg used 10 mM Tris-HCl, pH 7.5, with 5 mM EDTA to inhibit MMPs but allow the visualization of Plg activators. EC/collagen gels were also extracted in some cases to examine gelsolin expression as described (Salazar et al., 1999).

Reverse-transcriptase PCR (RT-PCR) analysis of differential gene expression

Cultures were established, total RNA was obtained and cDNA was synthesized as previously described (Salazar et al., 1999). A G3PDH primer set was utilized to show that equivalent amounts of cDNA were present in each sample (0, 8, 24, 48 hours) to standardize a stable expression pattern over the time course. The RT-PCR primer sets with their 5′-3′ sequences used for this study are listed below. For each gene pair, the first primer was the upstream primer while the second primer was the downstream primer.

MMP-1-ATTGGAGCAGCAAGAGGC; GTCCACATCTGCTCTTGGC

MMP-2-TGGCAGTGCAATACCTGAAC; CAAGGTCCATAGCTCATCGTC

MMP-3-CCTACTGTTGCTGTGCGTG; CAGCCTCTCCTTCATACAGC

MMP-9-GAGGAATACCTGTACCGCTATG; CAAACCGAGTTGGAACCAC

MT1-MMP-AAAGCAGCAGCTTCAGCC; CTCGGCAAAGAAGATCATG

TIMP-1-CACCAGAGAACCCACCATG; GCAGGCTTCAGCTTCCACTC

TIMP-2-TTTGCAATGCAGATGTAGTG; TCGAGAAACTCCTGCTTGG

PAI-1-ACCTCTGAGAACTTCAGGATGC; TTCACCCAGTCATTGATGATG

uPA-GACTCCAAAGGCAGCAATG; CGATGGTGGTGAATTCTCC

uPAR-ATTGCCGTGTGGAAGAGTG; GGTGTCGTTGTTGTGGAAAC

G3PDH-GCCAAAAGGGTCATCATCTC; GTAGAGGCAGGGATGATGTTC

Fluorescent collagen type I-degradation assay

Purified rat tail collagen was neutralized with NaOH and 10 mM $NaHPO_4$ to pH 7.5 at 0.25 mg/ml (total of 12.5 mg of collagen type I) and to this solution 1 mg of fluorescein isothiocyanate was added. This mixture was incubated at 4°C for 4-5 hours and was then dialyzed exhaustively using 0.1% acetic acid in H_2O. This sample was lyophilized, resuspended in 0.1% acetic acid at 5 mg/ml and was stored at 4°C. This fluorescent collagen preparation contained native type I collagen as indicated by its resistance to cleavage by trypsin as were our unlabelled collagen type I preparations. This fluorescent collagen was diluted 1:20 into 3.75 mg/ml collagen gels containing ECs. The cultures were established as described above except that Medium 199 was utilized that did not contain phenol red. Prior to the addition of the final medium containing growth factors, Medium 199 without phenol red but containing reduced serum supplement (100 μl) was added for 30 minutes at 37°C to deplete phenol red from the collagen gel mixture. This medium was then removed and replaced with Medium 199 without phenol red with complete additives as mentioned above. Conditioned medium was collected at various times and saved for measurements of fluoresence. Medium (100 μl) was placed into 96-well black Cliniplates (Labsystems, Helsinki, Finland) and fluoresence was determined using a FLUOstar 403 microplate fluorescence reader (BMG Lab Technologies, Durham, NC).

Construction of a recombinant adenovirus expressing the TIMP-1 cDNA

The full length human TIMP-1 cDNA was amplified by RT-PCR using KlenTaq polymerase (Clontech, Palo Alto, CA) from 8 hour EC cDNA (Salazar et al., 1999) using the primers listed above with added *Xho*I and *Eco*RV restriction enzyme sites in the upstream and downstream primers, respectively. The ~700 bp insert was unidirectionally cloned into pAdTrack-CMV (He et al., 1998) using *Xho*I-*Eco*RV digested vector and insert. Clones were sequenced to confirm the presence of the full length TIMP-1 cDNA and the absence of mutations. Preparation of plasmid recombinants with pAdEasy-1 were performed and virus was produced in human 293 cells as described (He et al., 1998). The pAdEasy adenoviral system was kindly provided by Drs Bert Vogelstein and Tong-Chuan He from Johns Hopkins University School of Medicine (Baltimore, MD).

RESULTS

Development of a novel in vitro model of capillary tube regression in three-dimensional collagen matrix following the addition of plasminogen

Using a previously described model of capillary morphogenesis in three-dimensional collagen matrices under serum-free defined medium conditions (Davis and Camarillo, 1996), we have examined the role of proteinases by adding plasminogen (Plg), which when converted to plasmin, can activate MMPs (He et al., 1989; Jeffrey, 1998). Initial experiments revealed that Plg addition did not effect the timing or extent of capillary tube formation. However, by 48 hours following Plg addition, the three-dimensional collagen gels markedly contracted (Fig. 1). This response was a highly reproducible event and occurred with collagen gels that consisted of 5 (data not shown) or 3.75 mg/ml (Fig. 1) of rat type I collagen. Because the latter dose of collagen was easier to manipulate in our assays (Salazar et al., 1999), we chose this concentration of collagen for the remaining experiments. The starting diameter of the collagen gels was 4.5 mm, while following contraction the gels were reduced to a diameter of ~ 0.5 mm. In Fig. 1, it can be seen that the addition of Plg did not affect EC morphogenesis prior to capillary tube regression (Fig. 1A vs B). However, with increasing time, capillary tubes in these cultures can be seen to undergo regression with retraction of EC tubes and processes resulting in aggregates of ECs and rounding of capillary tubes (Fig. 1C-F). Many individual ECs are rounded up and are no longer present within tube structures (Fig. 1E,F). The regression of capillary tubes and the rounding up of individual and groups of ECs occurs concurrently and progressively with the gel contraction process. The regression of tubes is clearly evident at the point where collagen gel contraction begins (Fig. 1C,D). After gel contraction, it is apparent that individual ECs are rounded up with no further evidence of morphogenesis (Fig. 1E,F).

The EC tube regression events appear to occur in part through an EC-mediated contractile mechanism. As shown in Fig. 2, a time course of EC-mediated contraction is observed on the same culture beginning at 37 hours of culture following Plg addition (Fig. 2A), and proceeding over a 9 hour period to fully contract (Fig. 2B-H). It should be noted that the entire

EC-collagen gel culture contracted into the highly compact state seen in Fig. 2H. Further experiments revealed that the EC contraction event required an active actin cytoskeleton. Cultures at the stage similar to that shown in Fig. 2A were treated with 40 μM cytochalasin B. The addition of cytochalasin B completely prevented the EC collagen gel contraction process (data not shown).

Evidence that endothelial cell apoptosis occurs during capillary tube regression in three-dimensional collagen matrices

Further support for the concept that this system is a model for capillary regression is that ECs appear to be undergoing apoptosis during this event. Preliminary experiments indicated that ECs were labelled by TUNEL assays in the Plg but not the control cultures (not shown). Using a flow cytometry apoptosis assay utilizing propidium iodide labelling (Maxwell and Davis, 2000a; Maxwell and Davis, 2000b) we have examined EC DNA content after 60 hour of culture with or without Plg addition. We observed an apoptotic rate of 28% in the Plg-containing cultures while only a 3% apoptotic rate was observed in the cultures without Plg. The apoptotic rate of 28% in the Plg-cultures is consistent with strong apoptotic responses that we have observed in previous studies of p53-mediated tumor cell apoptosis (Maxwell and Davis, 2000a; Maxwell and Davis, 2000b).

To more directly assess a role for apoptosis, evidence for caspase-dependent cleavage of proteins was found to occur during the regression process (Fig. 3). A well known target of caspase-3 is gelsolin (Kothakota et al., 1997; Kamada et al., 1998), an actin cytoskeletal regulatory protein, which we have previously shown is markedly upregulated in expression during capillary morphogenesis in three-dimensional collagen matrices (Salazar et al., 1999). Here, we show that 8-16 hrs after the initiation of collagen gel contraction, which occurred in this time course between 40-48 hours, that a specific cleavage of gelsolin occurred which was blocked by the caspase inhibitor, Z-VAD (Fig. 3). As shown in Fig. 3, gelsolin expression is induced during morphogenesis (with or without added Plg) but was selectively cleaved revealing a fragment of 45-50 kDa only during capillary regression. This result is consistent with previous reports of caspase-3 dependent cleavage of gelsolin (Kothakota et al., 1997). Furthermore, we have observed a similar decrease in intact 90 kDa gelsolin following the addition of anti-α2β1 integrin blocking antibodies (added at 40 hours of culture) but not following the addition of control anti-α5 blocking antibodies (Fig. 3, lower panels). Interestingly, the anti-α2 integrin antibodies induced marked regression of capillary tubes and rounding of individual ECs without inducing collagen gel contraction (Fig. 3). We have previously shown that α2β1 regulates the formation of tube structures in collagen type I matrices (Davis and Camarillo, 1996; Davis et al., 2000). These data indicate that Plg-dependent capillary tube regression induces an apoptotic response which is similar to that observed following the blockade of integrin-dependent

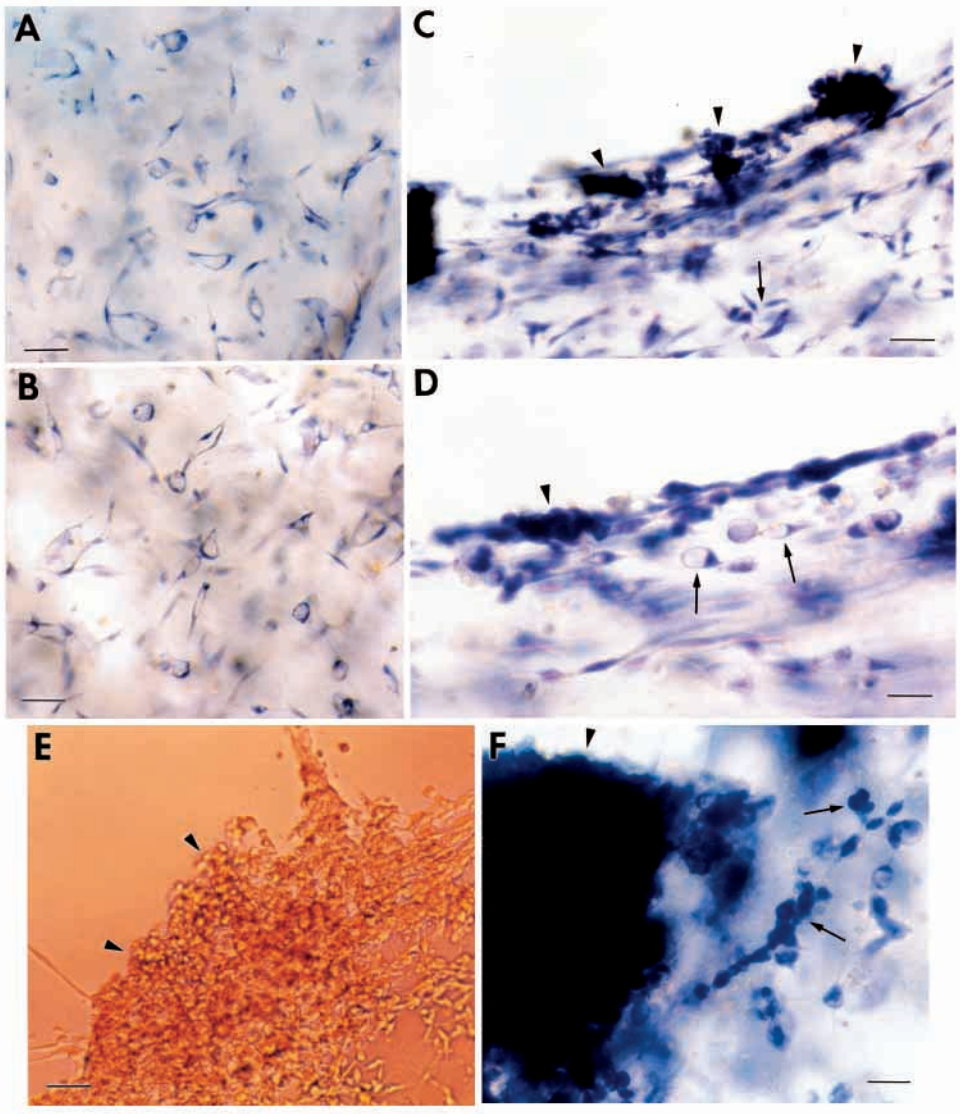

Fig. 1. Proteinase-dependent regression of capillary tubes in three-dimensional collagen matrices. Human ECs were suspended into three-dimensional collagen matrices as described (Davis and Camarillo, 1996) in the presence (A,C,D,E,F) or absence (B) of Plg and at various times of culture were fixed and stained. Cultures were fixed at 30 hours (A,B) before the beginning of regression or after various times of regression, 40 hours (C,D) or 46 hours (E,F). Arrowheads indicate clumps of regressing EC tubes which consist of ECs that are rounded up and aggregated together. Arrows indicate regressing capillary tubes (rounding up) (C,D) and small clusters of individual rounded up ECs (F). Bars: 100 μm (A,B); 50 μm (C,D,F); 250 μm (E).

survival signals (Meredith et al., 1993; Boudreau et al., 1996) and which also occurs following the blockade of the α2β1 integrin (Fig. 3).

We have also observed procaspase-3 cleavage (a reflection of caspase-3 activation) in the Plg-containing cultures consistent with an apoptotic response (Bossy-Wetzel and Green, 1999) while this was not observed in control cultures where a modest induction of caspase-3 protein was seen (Fig. 4A). The antibody used in this experiment does not detect cleaved/activated caspase-3. This time course of cleavage is earlier than that observed for gelsolin, a presumed caspase-3 target in this system (see Fig. 3), as decreased levels of procaspase-3 were detectable as early as 48 hours, which represents a time point several hours following full contraction of the collagen gels. In contrast, actin protein was essentially stable throughout in either set of cultures with or without Plg except at the 72 hour time point where a reduction was observed in the Plg containing culture. We have also observed a marked change in the electrophoretic mobility of another protein which is known to be proteolytically cleaved during apoptotic responses, p130cas (Kook et al., 2000). The p130cas protein, an adapter molecule and tyrosine kinase substrate in the focal adhesion complex, is required for integrin-dependent cell survival (Almeida et al., 2000). In Fig. 4, p130cas shows a reproducible change in its electrophoretic mobility by increasing its molecular mass in a time frame exactly corresponding to the timing of collagen gel contraction. This change is not observed in the cultures without Plg. In the time course shown in Fig. 4A, collagen gel contraction occurred between the 40 and 48 hour time points while in Fig. 4B, the contraction response occurred between the 44 and 48 hour time points in a separate time course. A clearly decreased mobility of p130cas on SDS-PAGE gels was observed in between these time points which indicates that this modification is a biochemical marker of the contraction and regression response. Interestingly, after this change occurs, the p130cas protein disappears within approximately 8 hours, a likely indication of proteolytic degradation. Interestingly, increased tyrosine phosphorylation of p130cas is observed directly corresponding to the timing of its decreased mobility in SDS-PAGE (Fig. 4B). Interestingly, the disappearance of p130cas is observed using both the p130cas and phosphotyrosine antibodies (Fig. 4A,B). In a number of different cell types, this property of slower electrophoretic mobility has been observed when the tyrosine phosphorylated state of p130cas is increased (Sakai et al., 1994).

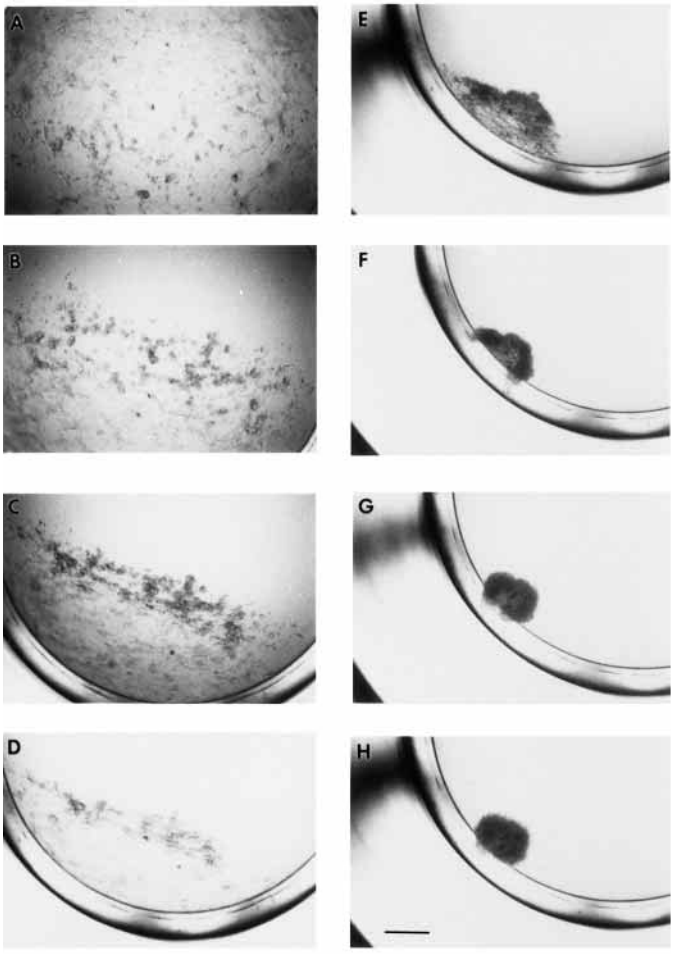

Fig. 2. Time course of plasminogen-induced endothelial cell contraction of three-dimensional collagen matrices. Human ECs were suspended into three-dimensional collagen matrices as described (Davis and Camarillo, 1996) in the presence of 2 μg/ml of Plg and at 37 hours of culture, a series of transmitted light photographs were taken of the same culture. (A) 37 hours; (B) 38 hours; (C) 39 hours; (D) 40 hours; (E) 41 hours; (F) 42 hours; (G) 45 hours; (H) 46 hours. Bar, 500 μm.

Matrix metalloproteinases and plasminogen activators are induced during endothelial cell morphogenesis in collagen matrices

To address whether MMPs or plasminogen activators (PAs) are induced during capillary morphogenesis to regulate the capillary regression process, we performed a series of time course experiments. We have previously shown that the RT-PCR technique allows for a convenient, semiquantitative method to examine the differential expression of mRNAs during capillary morphogenesis (Salazar et al., 1999). As shown in Fig. 5A, an RT-PCR experiment shows the expression of a series of proteinases as well as proteinase inhibitors in control cultures over time. Marked induction of mRNAs for MMP-1, MMP-3, MMP-9, MT1-MMP, and uPA were observed. The expression patterns of many of these genes have been confirmed at the protein level using gelatin zymogram and western blot analyses (see below). In contrast, MMP-2 mRNA was downregulated at 8 hours of culture only to be upregulated to near baseline by 48 hours of culture. Interestingly, TIMP-1 was upregulated at 8 hours and then downregulated to baseline by 48 hours while TIMP-2 was gradually downregulated over the 48 hour time period. Both PAI-1 and uPA receptor were upregulated at 8 hrs and then markedly downregulated by 48 hours. The expression of the control mRNA, G3PDH, was stable over the time course.

In Fig. 5B and C, gelatin zymograms show marked induction of a number of proteolytic enzymes during these events. Conditioned medium from control Plg-free cultures over time was collected and run on SDS-PAGE gels containing gelatin and Plg (to examine PAs) (Fig. 5B). Marked induction of uPA was observed while tPA also appeared to be induced but to a lesser extent than uPA (Fig. 5B). Other experiments examined MMP expression in the absence or presence of plasminogen

added to the culture medium (Fig. 5C) to investigate differences between control cultures versus cultures undergoing collagen gel contraction and capillary regression. In the control cultures, MMP-9 was shown to be induced as well as bands corresponding to MMP-2. An activated form of MMP-2 was observed to be markedly induced while pro-MMP-2 was present in conditioned medium as early as 4 hours and likely reflects constitutive synthesis of MMP-2 (Fig. 5C, upper panel). This conclusion was also supported by the mRNA expression pattern observed in Fig. 5A. In contrast, in the cultures with added Plg, MMP-9 appears to be processed into a lower molecular mass form indicative of its activation (Davis and Martin, 1990; Vu and Werb, 1998) while MMP-2 activation appears similar to that observed from cultures without added Plg. In addition, we have observed bands (~30 kDa) on these gels (indicated by the arrows) which are induced following Plg addition and during capillary regression events. The identity of these proteinases remains unclear as the activity of these bands was not inhibited by EDTA, serine protease inhibitors or various cathepsin inhibitors (not shown).

We have also performed western blot analysis to confirm the protein induction patterns for MMP-1 and MMP-9 (Fig. 6A). Dramatic induction of MMP-1 was observed, and interestingly, the addition of Plg resulted in the appearance of several lower molecular mass bands indicative of MMP-1 activation (Fig. 6A). The same result was observed with MMP-9 which is consistent with the gelatin zymogram results shown above (Fig. 5C). The antiserum utilized for the MMP-9 western blots also recognizes TIMP-1 since it was derived from rabbit immunizations with the MMP-9/TIMP-1 complex (Davis and Martin, 1990). Some induction of TIMP-1 protein was observed (Fig. 6A, lower panel) which is consistent with the RT-PCR data (Fig. 5A). Thus far, we have been unable to detect the production of MMP-3 on western blots despite evidence for induction of its mRNA using RT-PCR. Under the same conditions, we detected abundant MMP-3 release into conditioned medium by human fibroblasts (not shown).

Involvement of matrix metalloproteinases and plasminogen activators in plasminogen-induced contraction of collagen matrices and capillary tube regression

To determine a role for MMPs and/or PAs in Plg-induced capillary regression, experiments were performed using known inhibitors with specificity for different proteinases. The addition of aprotinin (a plasmin inhibitor), PAI-1 (a PA inhibitor), TIMP-1 and TIMP-2 (MMP inhibitors) and α2-macroglobulin (a broad spectrum protease inhibitor) completely inhibited Plg-induced collagen matrix contraction (Table 1). In contrast, the thrombin inhibitor, hirudin, had no effect. The results with TIMP-1 were also reproduced by overexpressing TIMP-1 in ECs using a recombinant adenovirus which prevented Plg-dependent collagen gel contraction and capillary regression while a control adenovirus had no effect (data not shown). Two low molecular mass inhibitors of MMPs, GM6001 (Galardy et al., 1994) and MMP inhibitor I (Odake et al., 1994), were also shown to inhibit the contraction process. Dose-response curves for each inhibitor were performed showing that complete inhibition was observed at doses above 50 nM for GM6001 (Fig. 6B) or above 16-32 μM for MMP inhibitor I (Fig. 6C). Also, western blots were performed to examine whether MMP-1

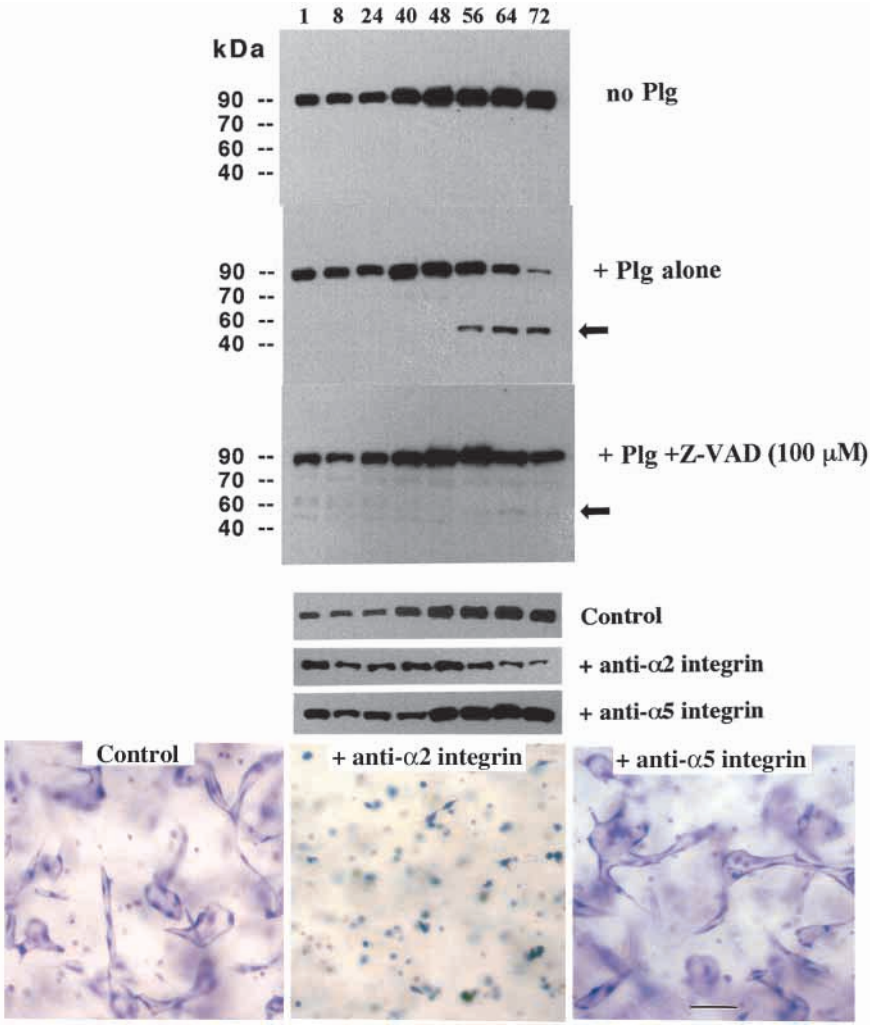

Fig. 3. Caspase-dependent cleavage of gelsolin occurs during proteinase-dependent capillary regression in three-dimensional collagen matrices. Human ECs were suspended into three-dimensional collagen matrices as described (Davis and Camarillo, 1996) in the presence or absence of 2 μg/ml of Plg and in the presence or absence of Z-VAD (100 μM). Extracts were made of EC cultures at the indicated times (three upper panels) and western blots were performed using anti-gelsolin antibodies. Arrows indicate the position of a gelsolin fragment at 45-50 kDa. The lower panel cultures were treated with either no antibody or 20 μg/ml of anti-integrin antibodies directed to the α2 or α5 subunits at 40 hours of culture. Extracts were made of EC cultures at the indicated times (middle panel) and western blots were performed using anti-gelsolin antibodies. Photographs of fixed and toluidine blue stained cultures treated with anti-integrin antibodies were taken 16 hours after addition (at 56 hours) (lower panels). Bar, 100 μm.

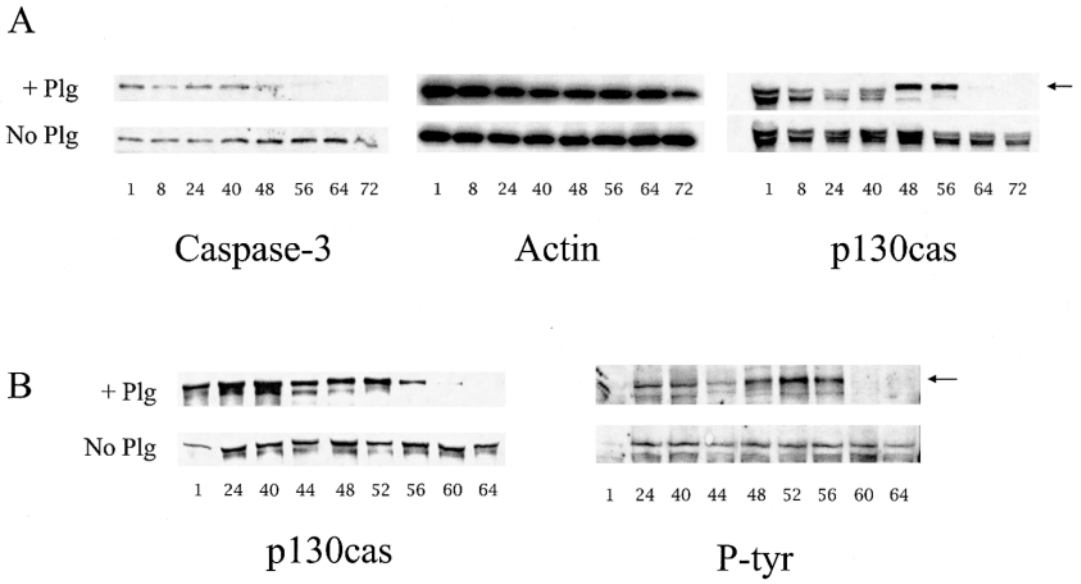

Fig. 4. Caspase-3 cleavage and alterations in p130cas occur during plasminogen-induced collagen gel contraction and capillary regression in three-dimensional collagen matrices. Western blots were performed using EC collagen gel culture (with and without added Plg) extracts prepared at the indicated times of cultures. (A) Blots used antibodies directed to caspase-3, actin, and p130cas. Arrow indicates the position of a p130cas band that migrates with a slower electrophoretic mobility. (B) Blots used antibodies directed to p130cas and phosphotyrosine. Arrow indicates the position of a p130cas and phosphotyrosine-containing band that migrates with a slower electrophoretic mobility.

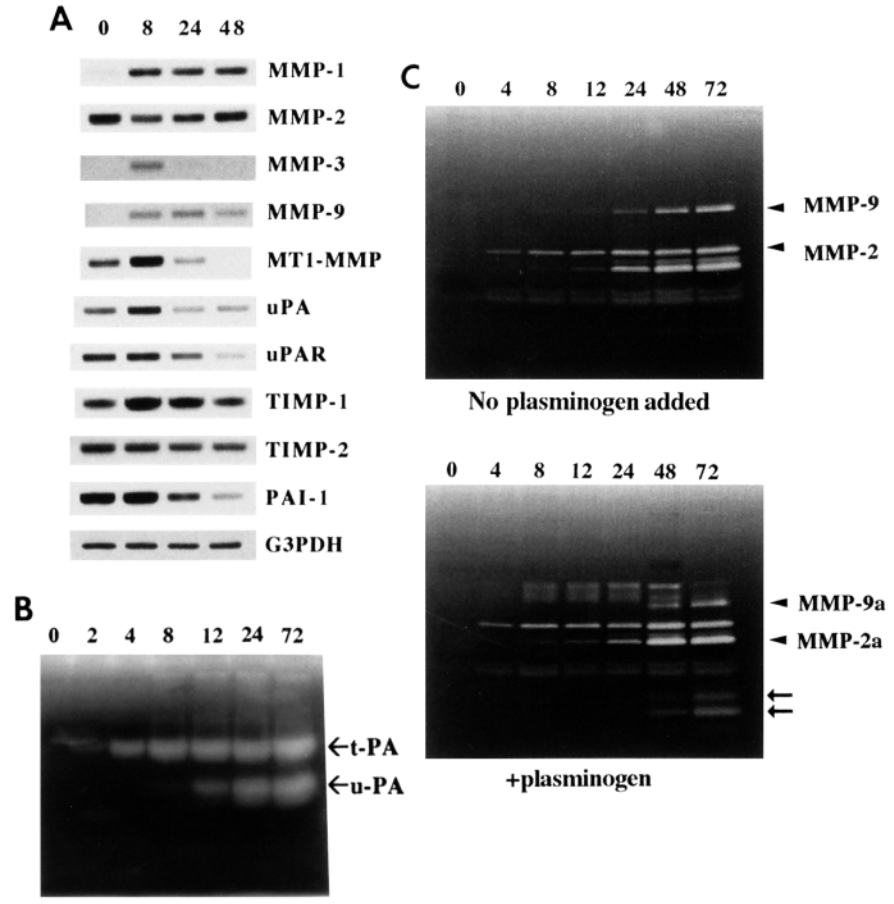

Fig. 5. Differential expression of matrix metalloproteinases, plasminogen activators, and proteinase inhibitors during capillary morphogenesis in three-dimensional collagen matrices. (A) Analysis of mRNA expression for proteinases and proteinase inhibitors during capillary morphogenesis in three-dimensional collagen matrices. Human ECs were suspended into three-dimensional collagen matrices, total RNA was obtained at 0, 8, 24 and 48 hours and semiquantitative RT-PCR was performed as described (Salazar et al., 1999). Samples were run on agarose gels and the PCR products were analyzed. The G3PDH product was used as a standard which showed equivalent amounts of starting cDNA for each sample as indicated by equivalent amounts of product in the four samples. (B) Gelatin zymography showing induction of plasminogen activators during capillary morphogenesis. Conditioned medium at different times of EC morphogenesis was loaded on SDS-PAGE gels containing gelatin and plasminogen. The position of the bands also corresponds to standards loaded for either t-PA or u-PA. (C) Gelatin zymography showing induction and activation of matrix metalloproteinases in endothelial cell-collagen cultures in the absence or presence of plasminogen. Human ECs were suspended into three-dimensional collagen matrices as described (Davis and Camarillo, 1996) in the presence or absence of 2 μg/ml of Plg. Conditioned medium was collected at various times of culture and was loaded on SDS-PAGE gels containing gelatin only. Arrowheads show MMP-9 and MMP-2 latent versus activated bands (MMP-9a, MMP-2a) while the arrows indicate bands observed which have not been identified.

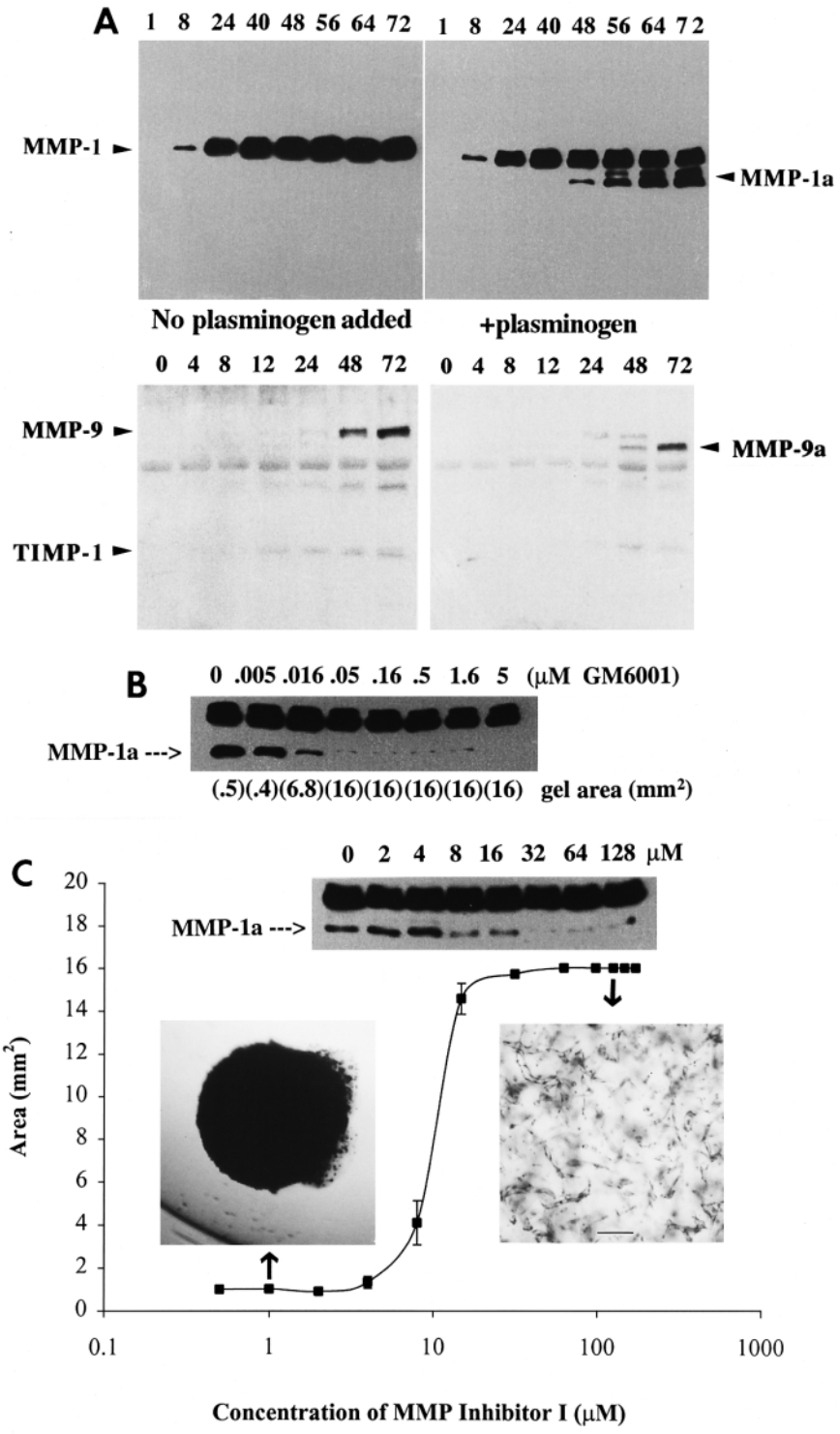

Fig. 6. Induction and activation of matrix metalloproteinases during capillary morphogenesis and following plasminogen addition. (A) Western blot analysis showing marked induction and activation of MMP-1 and MMP-9 during plasminogen-induced endothelial cell contraction of collagen matrices. Conditioned medium was collected at various times of culture and samples were loaded on 8.5% SDS-PAGE and after blotting to PVDF membranes, blots were probed with antibodies directed to MMP-1 (41-1E5) (upper panels) or the MMP-9/TIMP-1 complex (lower panels). The blots on the left were derived from conditioned medium obtained from cultures without Plg while the blots on the right were derived from medium obtained from cultures with added Plg. Arrowheads indicate the position of latent versus activated MMP protein bands. (B) Effect of MMP inhibitor GM6001 on plasminogen-induced collagen gel contraction. Varying doses of GM6001 were added to cultures in the presence of plasminogen and after 48 hours, cultures were fixed and conditioned medium was collected. Cultures were examined for collagen gel contraction (gel diameter averages of three independent gels are indicated in parenthesis under gel lanes) and medium was examined for MMP-1 activation by western blot analysis. Doses of GM6001 are indicated over the lane showing MMP-1 bands. Arrow indicates the position of an activated form of MMP-1. (C) Effect of MMP inhibitor I on plasminogen-induced endothelial cell contraction of collagen matrices. Human ECs were suspended into three-dimensional collagen matrices in the presence of 2 μg/ml of Plg and in the presence or absence of various doses of MMP inhibitor I. Cultures were fixed at 48 hours with glutaraldehyde and the area of the contracted or non-contracted gels was determined by measuring the average diameter (average of two perpendicular measurements) of triplicate gels. The data is expressed as the mean area ± standard deviation. Representative fields of the indicated doses (arrows) of MMP inhibitor I were photographed after fixation and toluidine blue staining. SDS-PAGE gels were run using conditioned medium samples derived from this dose response. The samples were run, blotted and probed with anti-MMP-1 antibodies to detect the bands. Arrows indicate the position of an activated form of MMP-1.

activation was blocked by the addition of these inhibitors. Interestingly, there was a direct correlation between the presence of activated MMP-1 and whether capillary regression occurred. In these dose-response curves, intermediate levels of activated MMP-1 correlated with partial gel contraction and regression (Fig. 6B,C). When MMP-1 activation was inhibited, EC morphogenesis occurred in a normal manner with no regression (Fig. 6C). There was also a direct correlation with inhibition of MMP-9 activation and lack of regression (data not shown).

An additional experiment addressed potential mechanisms regulating Plg activation during this process. A number of studies have shown that cell surface binding of Plg and the process of Plg activation is enhanced by proteins or peptides containing C-terminal lysine residues (Redlitz et al., 1995; Nesheim, 1998; Hajjar and Krishnan, 1999). To address whether such residues may play a role in the capillary regression process here, varying doses of the enzyme carboxypeptidase B were added to the culture medium. This

Table 1. Regression of capillary morphogenesis (collagen matrix contraction) in three-dimensional collagen matrices in response to plasminogen and various proteinase inhibitors

Proteinase inhibitor	Collagen matrix contraction	Gel area (mm^2)
None	+	0.32
Hirudin (1000 U/ml)	+	0.41
Aprotinin (910 KIU/ml)	–	16.0
Aprotinin (2900 KIU/ml)	–	16.0
PAI-1 (2.5 μg/ml)	–	16.0
PAI-1 (10 μg/ml)	–	16.0
TIMP-1 (1 μg/ml)	–	16.0
TIMP-1 (2.5 μg/ml)	–	16.0
TIMP-2 (1 μg/ml)	–	16.0
TIMP-2 (2.5 μg/ml)	–	16.0
α2-macroglobulin	–	16.0

Addition of plasminogen occurred at a culture time of 24 hours at 2 μg/ml and were measured 48 hours after plasminogen addition. Collagen gel areas were determined by calculating the average values from 4 independent cultures.

(–) No regression (gel area = 16 mm^2).

(+) Regression (gel area ≤0.5 mm^2).

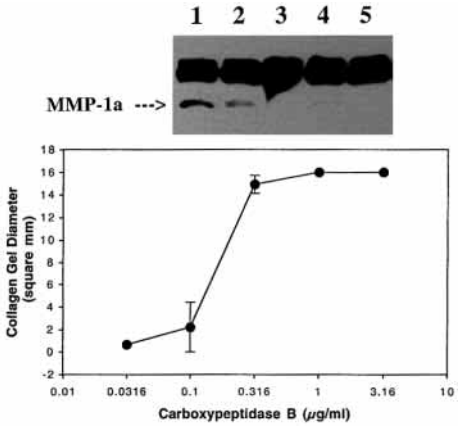

Fig. 7. Effect of carboxypeptidase B on plasminogen-induced endothelial cell contraction of collagen matrices. Cultures were established in the presence of 2 μg/ml of Plg and in the presence of varying doses of carboxypeptidase B. Cultures were fixed after 48 hours and were quantitated for collagen gel diameter. Triplicate cultures were examined and the values shown represent the mean ± standard deviation of these values. Conditioned medium samples derived from the dose-response curve were run on SDS-PAGE gels and western blots performed with anti-MMP-1 antibodies. Lane 1, 0.0316 μg/ml medium; lane 2, 0.1 μg/ml medium; lane 3, 0.316 μg/ml medium; lane 4, 1 μg/ml; lane 5-3.16 μg/ml. Arrow indicates an activated form of MMP-1.

enzyme degrades C-terminal lysine and arginine residues and has been reported to inhibit fibrinolysis through decreased plasmin generation (Redlitz et al., 1995; Nesheim, 1998). Carboxypeptidase B induced a dose-dependent inhibitory effect on Plg-dependent collagen gel contraction and capillary tube regression (Fig. 7). Interestingly, the addition of the enzyme also blocked MMP-1 activation (probably acting through decreased plasmin generation) showing again the correlation of capillary tube regression with the presence of activated MMP-1.

Table 2. Acceleration of endothelial cell-mediated collagen gel contraction and capillary regression by anti-PAI-1 blocking antibodies

Culture conditions	Collagen matrix contraction	Gel area (mm^2)
Plg alone	–	16.0
Plg + αPAI-2	–	16.0
No Plg + αPAI-1	–	16.0
Plg + αPAI-1	+	0.45
Plg + αPAI-1 + BSA	+	0.43
Plg + αPAI-1 + PAI-1	–	16.0
Plg + αPAI-1 + aprotinin	–	16.0
Plg + αPAI-1 + TIMP-1	–	16.0
Plg + αPAI-1 + TIMP-2	–	16.0
Plg + αPAI-1 + MMP inhibitor I	–	16.0

Addition of plasminogen and other reagents occurred at 0 hours of culture and cultures were fixed at 29 hours. The anti-PAI-1 and anti-PAI-2 polyclonal antibodies were added at 100 μg/ml. Gel areas were determined by measuring the areas of three independent cultures and calculating the average. In addition, proteinase inhibitors were added at the following concentrations. Aprotinin, 2900 KIU/ml; PAI-1, 10 μg/ml; TIMP, 1- 2.5 μg/ml; TIMP-2, 2.5 μg/ml; MMP inhibitor I, 175 μM.

Endogenous production of PAI-1 by endothelial cells negatively regulates plasminogen-induced capillary tube regression and collagen gel contraction

As shown in Fig. 5A, a series of EC proteinase inhibitors were found to be differentially regulated during EC morphogenesis. To address whether EC-derived PAI-1 played a role in regulating the Plg-induced contraction event, blocking antibodies were added. As shown in Table 2, Plg-induced contraction occurred more rapidly (within 24-29 hours) in the presence of both Plg and blocking antibodies to PAI-1 than in controls with Plg alone. In contrast, no effect was observed with control blocking antibodies directed to PAI-2. In addition, the ability of anti-PAI-1 antibodies to accelerate regression was only observed in the presence of Plg and not in its absence (Table 2). This response was even more rapid when anti-PAI-1 and Plg were added after 24 hours of culture, as contraction was observed at 12-16 hours following addition (data not shown). The accelerated contraction response with anti-PAI-1 antibodies was blocked by the addition of the proteinase inhibitors, PAI-1, aprotinin, TIMP-1, TIMP-2 and MMP inhibitor I indicating that the effect was both plasmin and MMP-dependent (Table 2).

Blocking antibodies to MMP-1, MMP-9 and MMP-2 interfere with plasminogen-induced capillary regression and contraction of collagen matrices

As shown above, marked increases in the production of MMP-1 and MMP-9 occurred during EC morphogenesis as well as marked increases in the presence of activated forms of MMP-2 (with or without Plg), MMP-1 (only with Plg) and MMP-9 (only with Plg). To address whether these activated MMPs directly participated in the process of Plg-induced contraction of collagen matrices, blocking monoclonal antibodies were added during the process. As shown in Fig. 8, blocking antibodies directed to MMP-1, MMP-2 and MMP-9, all independently blocked collagen gel contraction. In contrast, a non-blocking antibody to MMP-1 as well as a control anti-integrin antibody to the α5 subunit, had no blocking effect. The addition of the blocking MMP-1 antibody interfered with the

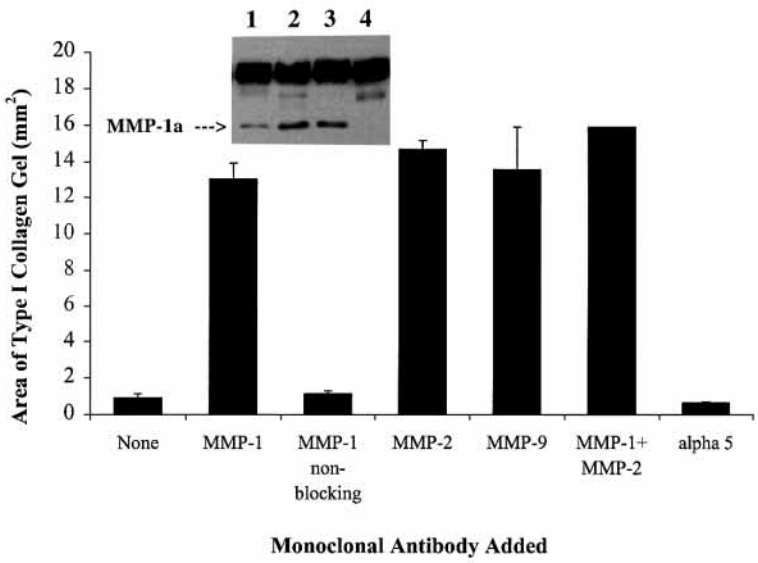

Fig. 8. Effect of blocking monoclonal antibodies directed to matrix metalloproteinases on plasminogen-induced endothelial cell contraction of collagen matrices. Human ECs were suspended into three-dimensional collagen matrices as described (Davis and Camarillo, 1996) in the presence of 2 μg/ml of Plg and in the presence or absence of 20 μg/ml of monoclonal antibodies directed to various MMPs and the α5 integrin. In one case, two different antibodies were mixed. Cultures were fixed at 48 hours with glutaraldehyde and the area of the contracted or non-contracted gels was determined by measuring the average diameter (average of two perpendicular measurements) of triplicate gels from two independent experiments. The data is expressed as the mean area ± standard deviation. The figure inset shows an anti-MMP-1 western blot of conditioned medium of several of these cultures. The SDS-PAGE gel was run under non-reducing conditions. The arrow indicates an activated form of MMP-1. Lane 1, non-blocking anti-MMP-1 antibody medium; lane 2, control α5 integrin control antibody medium; lane 3, control medium – no antibody added; lane 4, blocking anti-MMP-1 antibody medium.

generation of activated MMP-1, while control antibodies did not, accounting for its effects on collagen gel contraction (Fig. 8). These data show that at least three MMPs directly participate in this contraction and regression process.

Evidence that collagen degradation is the mechanism for matrix metalloproteinase-induced collagen gel contraction and capillary tube regression

To address whether the induction and activation of MMPs results in detectable collagen type I degradation, experiments were performed using a fluorescent collagen type I degradation assay. For these experiments, type I collagen was labelled with fluorescein and incorporated into the three-dimensional collagen assay. Cultures were established and conditioned medium was collected at various times and assayed for fluorescence using a fluoresence microplate reader. In Fig. 9A, it is shown that a marked fluorescence release was detectable in the culture medium only following Plg addition and subsequent contraction. This release of fluorescent collagen was observed between 24 and 48 hrs, corresponding to the timing of the contraction event. In addition, this coincides with the temporal time frame when activated forms of MMP-1 and MMP-9 were detected (see Fig. 6A). We observed that release of fluorescent collagen degradation products only occurred following the addition of Plg. This fluorescent release accounted for 32±8% (*n*=4) of the total fluorescent material present in the collagen gels. The addition of the proteinase inhibitors, aprotinin, PAI-1, TIMP-1 and TIMP-2 completely blocked fluorescent collagen release (Fig. 9B). These data show that fluorescent release of collagen degradation products directly correlates with Plg addition and capillary regression, and is blocked by plasmin, PA and MMP inhibitors. These data suggest that collagen degradation by EC proteinases regulates the contraction and regression process.

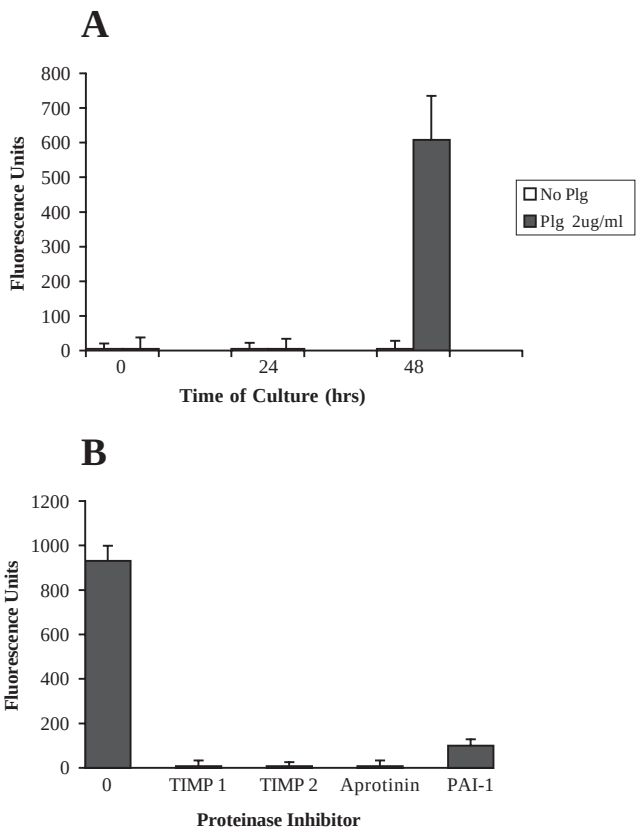

Fig. 9. Fluorescent collagen degradation and release during plasminogen-induced endothelial cell contraction of collagen matrices. Collagen type I was labelled with fluorescein and was incorporated into the endothelial cell-collagen gel mixture. The cultures were established in the presence or absence of added Plg using Medium 199 culture medium without phenol red. At various times of culture, conditioned medium was collected and assayed for fluorescence using a microplate fluorescent reader. (A) Fluorescence readings (average ± standard deviation) from six independent cultures are shown for 0, 24 and 48 hours cultures in the presence or absence of Plg. (B) Additional experiments collected conditioned medium from cultures containing proteinase inhibitors (48 hours). Fluorescence readings (average ± standard deviation) from six independent cultures cultured in the presence of Plg in the presence or absence of the indicated proteinase inhibitors.

DISCUSSION

Plasminogen induces matrix-metalloproteinase-dependent regression of capillary tube networks in three-dimensional collagen matrices

Previous work has shown that Plg is an important molecule regulating wound healing responses (Pepper et al., 1996; Clark, 1996; Mignatti et al., 1996; Carmeliet and Collen, 1998; Lund et al., 1999). It is also the precursor of plasmin, an important fibrinolysin, as well as a known activator of MMPs such as MMP-1, MMP-3, and MMP-9 (He et al., 1989; Okumura et al., 1997; Jeffrey, 1998; Murphy and Gavrilovic, 1999; Ramos-DeSimone et al., 1999). In previous studies, Plg has been shown to play a role in inducing regression of blood vessels. A fragment of Plg, termed angiostatin, has been shown to inhibit angiogenesis and affect tumor growth (O'Reilly et al., 1994). Angiostatin has been shown to influence a number of EC properties related to angiogenic responses such as proliferation, apoptosis and migration (O'Reilly et al., 1994; Lucas et al., 1998; Redlitz et al., 1999). The data presented here support the hypothesis that Plg caused capillary regression by its conversion to plasmin, which caused MMP activation and subsequent collagen matrix degradation. Whether angiostatin can influence plasmin generation or MMP activation is unclear at present, although one study suggests that angiostatin may inhibit plasmin generation at the cell surface by competing for Plg cell surface-binding sites (Stack et al., 1999). The binding of Plg to ECs and other cell types has been shown to involve C-terminal lysine residues in cell surface Plg-binding proteins such as annexin II (Hajjar and Krishnan, 1999). It seems likely that our results represent a distinct mechanism for Plg-induced capillary regression compared to that reported for angiostatin since it does not have intrinsic proteolytic activity (O'Reilly et al., 1994).

Matrix metalloproteinases are induced and activated during endothelial cell morphogenesis and directly participate in plasminogen-induced capillary regression in three-dimensional collagen matrices

The work presented here shows that a number of proteolytic enzymes are differentially expressed during EC morphogenesis in collagen matrices which is consistent with previous studies (Mignatti et al., 1989; Fisher et al., 1994; Cornelius et al., 1995; Iruela-Arispe et al., 1995; Pepper et al., 1996; Haas et al., 1998; Hiraoka et al., 1998; Werb et al., 1999). We provide evidence for induced activation of MMP-1, MMP-2, and MMP-9. Interestingly, activated states for MMP-1 and MMP-9 were only observed following Plg addition, while MMP-2 was similarly activated with or without Plg addition, likely due to activation by MT-MMPs (Will et al., 1996; Foda et al., 1996; Deryugina et al., 1998; Knauper and Murphy, 1998). Despite the presence of activated MMP-2 in cultures without Plg, activated MMP-2 alone is not sufficient to induce EC-mediated collagen gel contraction. Plasmin-mediated activation of MMP-1 and MMP-9 is required in conjunction with activated MMP-2 for this event.

Our data with blocking monoclonal antibodies to MMPs suggests that MMP-1, MMP-2 and MMP-9 all participate and are independently required for Plg-induced collagen gel contraction. The substrate specificities of these three MMPs are different (Vu et al., 1998; Jeffrey, 1998; Yu et al., 1998). In particular, MMP-1 is efficient in degrading native collagen type I, while MMP-2 and MMP-9 are very efficient in degrading denatured collagen type I. Perhaps the degradation of both native and denatured collagen type I are necessary for collagen gel contraction and thus, capillary regression.

Endothelial cell apoptosis occurs during matrix metalloproteinase-dependent capillary regression in three-dimensional collagen matrices

The data presented here also supports the concept that matrix metalloproteinase-dependent capillary regression in three-dimensional collagen matrices leads to EC apoptosis. This information further validates our system as a model for capillary tube regression. Western blot experiments provided evidence for caspase-dependent cleavage of the known caspase-3 target, gelsolin, as well as caspase-3 itself, during plasminogen-dependent capillary regression. In addition, anti-α2 integrin antibodies which cause marked regression of pre-existing capillary tubes (Fig. 3), also showed marked decreases in intact gelsolin in a time course similar to that of matrix metalloproteinase-induced regression (Fig. 3). These data support the concept that EC apoptosis is occurring secondary to decreased or altered cell-matrix contacts leading to cell rounding, a feature common to both the effects of Plg or anti-integrin antibody addition (see Figs 1 and 3). Loss of cell-matrix contacts is well known to induce apoptosis in ECs and other cells (Meredith et al., 1993; Boudreau et al., 1996; Werb et al., 1996). In support of such conclusions was the observation that alterations in p130cas occurred during the Plg-induced regression response. The p130cas protein is an important adapter molecule and tyrosine kinase substrate in the focal adhesion complex necessary to support cell survival in response to cell-ECM signals (Almeida et al., 2000). Decreased electrophoretic mobility of p130cas was seen along with increased tyrosine phosphorylation of the protein which directly correlated temporally with the contraction and regression events. This data suggests that alterations in signals derived from the focal adhesion complex may be an important component of the apoptotic response in this system. The proteolytic removal of the p130cas protein within an 8 hour period following this mobility shift supports this idea.

A novel in vitro model for endothelial cell-mediated collagen gel contraction and regression of capillary tubes in three-dimensional collagen matrices

The work described here presents a novel in vitro model for capillary tube regression that involves MMP activation and depends on MMP-dependent proteolysis of collagen matrices. Many in vitro studies have focused on the question of how capillary tubes assemble in three-dimensional ECM environments (Montesano and Orci, 1985; Vernon and Sage, 1995; Pepper et al., 1996; Davis and Camarillo, 1996; Bayless et al., 2000). In contrast, few studies have investigated mechanisms of capillary tube regression in three-dimensional ECM (Zhu et al., 2000). Much recent work has concentrated on molecules or mechanisms to induce vascular regression in vivo as a way to inhibit tumor survival, growth and progression (Brooks et al., 1994; Senger et al., 1997; O'Reilly et al., 1994; O'Reilly et al., 1997; Browder et al., 2000). However, the complexity of these in vivo systems make it difficult to identify the molecular pathways which regulate these phenomena.

To address these issues, we developed a highly reproducible microassay to study the molecular regulation of human capillary tube regression. This regression mechanism directly involves two apparently related phenomena which are EC-mediated collagen degradation and EC-matrix contractile behavior. Several previous studies have revealed that collagen gel contraction by various cells can be inhibited by proteinase inhibitors (Deryugina et al., 1998; Vernon and Sage, 1996; Myers and Wolowacz, 1998; Scott et al., 1998). It should be pointed out that the collagen gels utilized in our studies are very dense (3.75 or 5 mg/ml collagen type I) compared to many previous studies concerning cell-mediated collagen gel contraction (usually 1-1.5 mg/ml) (Schiro et al., 1991). The ability of ECs to contract gels of this density is quite remarkable suggesting either a marked ability to generate cell-matrix contractile forces and/ or to degrade collagen matrices. Previous studies have shown that ECs can mechanically distort ECM which has effects on the ability of ECs to undergo morphogenesis and organize into planar cord structures (Vernon et al., 1992; Vernon and Sage, 1995; Davis and Camarillo, 1995). It is also important to consider that ECs undergoing morphogenesis have formed interconnected multicellular structures (i.e. networked tubes) which may allow increased generation of mechanical forces compared to individual cells (i.e. fibroblasts) interacting with ECM. An interesting question is the extent to which ECs undergoing angiogenesis in a wound site may participate in the known phenomenon of wound contraction, since there is some overlap in the time frames for capillary regression and wound contraction during wound healing (Clark, 1996).

In addition, blood vessel formation is followed in a controlled temporal manner by blood vessel regression in the normal wound healing process (Clark, 1996; Madri et al., 1996). When this process is abnormal, such as in chronic wounds, diabetic retinopathy or cancer, this vasculature remains and does not regress suggesting that normal vascular regression responses may be circumvented. One possibility is that regression inhibitors are present in these situations which prevent the normal regression process. The data presented with EC-derived PAI-1 negatively regulating Plg-induced contraction illustrates this point. Blocking antibodies to PAI-1 markedly accelerated the regression and contraction process (see Table 2). Also in support of this idea, are recent in vivo studies implicating a role for PAI-1 in the prevention of vascular regression and in the facilitation of tumor growth and angiogenesis (Bacharach et al., 1998; Bajou et al., 1998). Furthermore, recent data indicate that PAI-1 is a hypoxia-induced gene along with VEGF (Pinsky et al., 1998) indicating that inhibition of the proteinase-induced regression mechanism described here may be an important feature of angiogenic responses.

Two classes of proteinases controlling the collagen gel contraction and regression response presented here are plasmin (generated from Plg and PAs) and MMPs (MMP-1, MMP-2 and MMP-9). It is interesting that these two classes of enzymes can either separately or in combination degrade both fibrin and collagen matrices, which are the most biologically relevant ECM environments where blood vessel formation and regression take place (Vernon and Sage, 1995; Senger, 1996; Pepper et al., 1996). Thus, our work strongly implicates plasmin and MMP-mediated degradation of extracellular matrix as a major mechanism controlling regression of capillary tube networks.

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